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CITATION:

Mori, K ...[et al]. Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage.. Kidney international 2007, 71(10): 967-970

ISSUE DATE:

2007-05

URL:

http://hdl.handle.net/2433/156871

[Title]

Neutrophil gelatinase-associated lipocalin as the real-time indicator of active kidney damage

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[Running Headline]

Ngal and renal failure

ABSTRACT

Neutrophil gelatinase-associated lipocalin (Ngal, 24p3, SIP24, lipocalin 2 or siderocalin) was originally purified from neutrophils but with unkown function. Recently, it was identified that Ngal activates nephron formation in the embryonic kidney, is rapidly and massively induced in renal failure and possesses kidney protective activity. We would like to propose that blood, urine and kidney Ngal levels are the real-time indicators of active kidney damage, rather than one of many markers of functional nephron number (as Forest Fire Theory). Ngal is a novel iron carrier protein exerting pleiotropic actions including the upregulation of epithelial marker E-cadherin expression, opening an exciting field in cell biology.

KEY WORDS: progression of renal failure; development; iron; siderophore; mesenchymal-epithelial transition; ischemia-reperfusion; gene expression.

MOLECULAR STRUCTURE OF NGAL

Neutrophil gelatinase-associated lipocalin (Ngal) belongs to the lipocalin superfamily, which are secreted or cytosolic proteins with barrel-like structure, carrying hydrophobic ligands (such as fatty acids, retinoids and pheromones) in their core pocket.[1] Recent works have elucidated the pathophysiological roles of lipocalins in energy homeostasis. Retinol binding protein 4 impairs insulin signaling in muscles causing insulin resistance,[2] while lipocalin-type (or brain) prostaglandin D synthase (PGDS or beta-trace) inhibits insulin-stimulated proliferation of vascular smooth muscle cells resulting in protection against atherosclerosis.[3] Mice with a null mutation in adipocyte fatty acid-binding protein (FABP4 or adipocyte protein aP2) become obese under high-fat diet but do not develop insulin resistance.[4] Little is known about the involvement of the ligands in these metabolic activities of lipocalins. Goetz and Strong et al. carried out an epoch work of the X ray christallography for recombinant human Ngal protein expressed in E. coli.[5] They demonstrated that the ligand for Ngal is siderophore. Siderophores are a diverse group of small (1kDa or less) non-peptide iron (Fe^{3+})-binding chemicals produced in bacteria, fungi and plants, and their mammalian version remains to be identified.[6-9] Of note, XL1-Blue strain of E. coli synthesizes a siderophore called enterochelin (or enterobactin), but BL21 commonly used for recombinant protein expression does not make enterochelin.[5] Probably outside the pocket, Ngal binds with gelatinase B (matrix metalloproteinase-9 or type IV collagenase) and with hepatocyte growth factor and modulates their activity.[10-12]

NGAL IN KIDNEY DEVELOPMENT

Mammalian metanephric mesenchyme and ureteric bud (UB) coordinate a complicated interaction to develop themselves into mature nephrons.[13] Barasch et al. established an organ culture system where isolated rat metanephric mesenchyme converts into glomeruli and proximal tubules by stimulation with condition media prepared from a mouse UB cell line.[14] Leukemia inhibitory factor (LIF) and Ngal were the epithelial inducers purified at the protein level using this assay.[14,15] Genetic inactivation of Ngal (by Flo et al.) or LIF signal transducer gp130 does not result in agenesis of the kidney, indicating a high redundancy in nephrogenesis pathways.[14,16] Yang, Mori et al. have shown that mouse Ngal protein secreted from cultured UB cells possesses nephron-inducing activity and also can bind iron.[15] Surprisingly, Ngal:bacterial siderophore:iron complex has much stronger activity compared to Ngal:siderophore (without iron) and apo-Ngal (without siderophore),[17] indicating the first example of "siderophore-binding proteins," in any species, dependent upon the presence of iron for

the biological activity.[7]

NGAL IN RENAL FAILURE

Recapture of genetic program of embryo is often observed in tissue injuries. Devarajan et al. have analyzed animal models of acute renal failure to screen biomarkers useful in the clinical settings and to understand the molecular mechanisms of kidney injury, to begin with by use of microarray.[18,19] Within a few hours, Ngal mRNA is highly upregulated after kidney injury, such as renal ischemia-reperfusion and cisplatin nephropathy, where Ngal induction precedes the elevation of classical markers for kidney damage: serum creatinine, urinary N-acetyl glucosaminidase and beta 2-microglobulin levels.[19,20] Furthermore, Mori et al.[9] and other groups have reported that Ngal protein is abundantly accumulated in the blood, urine and renal proximal and distal tubules in acute renal failure of humans: in cases associated with renal ischemia (sepsis, hypovolemia, heart failure), nephrotoxin (antibiotics, cisplatin, bisphosphonate, nonsteroidal anti-inflammatory drugs, radio-contrast, hemoglobinuria), kidney-parenchymal damage (glomerulonephrits, minimal change disease, focal segmental glomerulosclerosis, diabetic nephropathy), hemolytic-uremic syndrome and post-transplant rejection. Mishra and Mori et al. presented the clinical usefulness of blood and urinary Ngal as extremely early markers of acute kidney disease.[21] As early as 1-2 hours after the cardiopulmonary bypass surgery in children and in adults (with average bypass time of 2-3 hours), the Ngal levels are dramatically and specifically elevated in those who are going to develop acute renal failure (diagnosed by more than 50% rise in the serum creatinine levels a few days later).[21,22] Acute worsening of chronic renal failure may be also predicted by elevated urinary Ngal levels (Nickolas, Mori, Barasch et al. submitted). In general, to evaluate the severity of renal failure, at least two aspects should be considered (Figure 1): the ratio of functional versus atrophic nephrons (or the results of kidney injury) and the severity of on-going damage. Analogy may hold true for forest fire (Forest Fire Theory). We would like to propose here that induction of Ngal expression is a real-time indicator of active renal injury.

Functional significance of Ngal upregulation in renal failure was investigated by Mori and Mishra et al., who analyzed separate sets of animals.[9,23] Single intraperitoneal or subcutaneous injection of recombinant Ngal protein into mice (Figure 2) significantly ameliorates kidney damage after renal ischemia-reperfusion injury, if Ngal is given before ischemia or 1 hour after reperfusion. Berger et al. generated Ngal kockouts and found no difference in renal damage at 24 hours after renal ischemia-reperfusion compared to wild-type mice.[24] Although Ngal mRNA upregulation

is a rapid response, the speed and amount of endogenous Ngal induction may not be sufficient to show significant protection in this setting. Experiments in more chronic and milder models may give different results.

TRANSCRIPTIONAL REGULATION OF NGAL

Neutrophils, [25] monocytes/macrophages[26] and adipocytes[27] are cells with abundant Ngal expression (Figure 3). Importantly, immature neutrophils (myelocytes and metamyelocytes) have high expression level of Ngal mRNA, while mature neutrophils/granulocytes in the circulation have lost the mRNA but contain large amount of Ngal protein,[25] making it impossible to determine the involvement of neutrophil-dereived Ngal in tissue injury by in situ mRNA hybridization. Ngal expression is highly induced not only in kidney injury, but also in epithelial inflammation of intestine,[28] skin and airway,[29] and in bacterial infection[16] and cancer.[30] Ngal inducers so far identified in vitro are interleukin-1 beta, tissue necrosis factor alpha, lipopolysaccharide, basic fibroblast growth factor, prostaglandin F2 alpha, phorbol ester, dexamethasone, retinoic acid, serum and hypoxia.[20,26,29,31]

BIOLOGICAL ACTIVITY OF NGAL

Ngal exerts a broad range of biological activities (Figure 3). The co-existence of siderophore and iron is required not only for mesenchymal-epithelial transition (MET) of embryonic kidney[17] and oncogene Ras-transformed epithelial cells (by Hanai and Sukhatme et al.)[30] but also for kidney protection from renal failure (by Mori et al.).[9] On the other hand, Ngal:siderophore complex without iron[17] (and potentially apo-Ngal) [32] can chelate iron from cells and iron deprivation is the mechanism for apoptosis of pro-B cells,[32,33] and for inhibition of bacterial growth and erythropoiesis.[5,16,34] Various other activities of Ngal are increasingly reported.[12,28]

Induction of MET by Ngal is associated with upregulation of epithelial marker E-cadherin expression, but this is a slow process. In metanephric mesenchyme, morphologically distinct epithelia is observed only 7-10 days after addition of Ngal:siderophore:iron complex (unpublished observation).[15] By contrast, epithelia induced by LIF can be observed within 4-5 days.[13,14] In Ras-transformed mammary cells, E-cadherin protein accumulation was evaluated 48 hours after treatment with the complex.[30] Hanai and Mori et al. demonstrated that treatment with Ngal:siderophore:iron suppresses Raf-MEK1/2-ERK1/2 pathway of mitogen-activated protein kinase and inhibits E-cadherin phosphorylation, causing decrease in E-cadherin degradation.[30] Not only treatment with Ngal:siderophore:iron complex but also

transfection with cDNA and infection with adenovirus encoding Ngal results in MET of transformed cells, implying that fetal calf serum or cells themselves provide mammalian siderophores. In the case of renal ischemia-reperfusion injury, phosphorylation of MEK1/2 and ERK1/2 is biphasic (transient activation within 30 min, followed by long term activation for several days) and the levels of their phosphorylation are not necessarily parallel with the severity of renal injury,[35] suggesting that suppression of this pathway is not the major mechanism for Ngal-mediated kidney protection. Administration of Ngal is ineffective if given 2 hours after reperfusion,[9] implying that Ngal is preventing the early injuries (in part by upregulating a protective enzyme heme oxygenase-1)[9] rather than stimulating the recovery process.

In healthy adult kidneys, Ngal[36] and other lipocalins (whose sizes are 17-43 kDa) including retinol-binding protein 4, alpha 1-microglobulin,[37] and probably also liver-type fatty acid-binding protein (FABP1) and PGDS are freely filtrated in the glomeruli, bound to multi-ligand scavenger receptor megalin (expressed abundantly and specifically on the brush borders of proximal tubules) and taken up efficiently by endocytosis (as well as albumin and beta 2-microglobulin).[38] Insufficient tubular reabsorption (due to specific saturation in the endocytic pathway or general malfunctioning of proximal tubules) should contribute in part to urinary Ngal. Devireddy et al. identified brain type organic cation transporter as Ngal receptor in pro-B cells.[32]

Iron-free siderophore-like activity (which assists the binding of Ngal and Fe^{3+}) is detectable in the normal urine of humans, mice, rats and dogs.[9,39] Tear lipocalin, a major protein component especifically found in human tears, also binds with siderophores.[40] Lipocalin 12, found specifically in mouse epididymis, is also structurally related to Ngal and may have binding capacity for siderophores.[41]

CHARACTERISTICS OF NGAL AS RENAL FAILURE BIOMARKER

Ngal induction is a rapid event detectable within a few hours, characterizing Ngal as one of immediate early genes or acute phase reactants such as interleukin-6 and C-reactive protein. Fold induction of Ngal mRNA and protein is log order of magnitude, reaching 1000-fold in most severe cases of renal injury.[9,39] Therefore, normalization for urinary creatinine level is not necessary to evaluate urinary Ngal.[21] Indeed, a patient with urinary Ngal level of 40 microgram/ml died of multi-organ failure 12 hours after urine collection (Mori, Kunis et al., unpublished observation).[9]

DIRECTIONS FOR FUTURE RESEARCH

There are a number of siderohpre-dependent and –independent activities reported for Ngal. The role of endogenously expressed Ngal in these activities should be better verified using Ngal deficient mice. During renal ischemia-reperfusion injury, the liver also starts to express Ngal mRNA.[39] Therefore, the source of Ngal protein in the blood and urine during renal injury can be a complex from the kidney, liver and white blood cells (in the injured tissues and in the circulation). Tissue-specific disruption of Ngal gene will make it possible to investigate what is the predominant source of Ngal, as a biomarker or as a protective mechanism for renal failure. Purification and identification of mammalian siderophores, and to learn their metabolism and regulation are also important steps. The reported method to recognize siderophores is only available for iron-free molecules.[9] A new way to detect iron-loaded siderophores must be invented. Characterization of Ngal receptors, their downstream intracellular signaling and subcellular localization of events will help elucidate the detailed molecular mechanism underlying the actions of Ngal, siderophores and iron.

CONCLUSION

Ngal is a very unique protein endowed with iron carrying activity and diagnostic and therapeutic utilities for renal failure, which is acquiring an explosion of attention by researchers and clinicians beyond nephrology.

ACKNOWLEDGMENTS

Unfortunately, I could not describe all the contributors and original papers in Ngal research. I would like to especially acknowledge Drs. J. Barasch, P. Devarajan and Q. Al-awqati for discussion, instruction and encouragement. I am also grateful to Drs. J. Yang, K.M. Schmidt-Ott, J.Y. Li, N. Paragas, A. Kalandadze, H.T. Lee, F.H. Cheema (Columbia University) and M. Mukoyama, M. Kasahara, H. Yokoi, K. Sawai, T. Suganami and T. Kuwabara (Kyoto University). This work has been supported by grants from Uehara Memorial Foundation, Yamanouchi Foundation for Research on Metabolic Disorders, Salt Science Research Foundation, Japanese Ministry of Education, Culture, Sports, Science and Technology, and from Japan Society for Promotion of Science.

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FIGURES

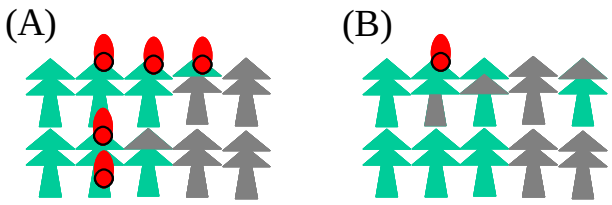

Figure 1. Forest Fire Theory for worsening renal function.

Forest (kidney) is composed of trees (nephrons). Both model A and B have 60% viable trees (shown in green), and 40% of trees are burnt down (shown in gray, corresponding to sclerosis of glomeruli and atrophy of tubules). However, model A has much stronger fire (shown in red, that is ongoing nephron damage) than model B. We would like to propose that serum creatinine level or glomerular filtration rate is a marker for functional nephron numbers (green trees), whereas serum, urinary or renal Ngal level indicates the extent of active lesion in the kidney (red fire in the forest).

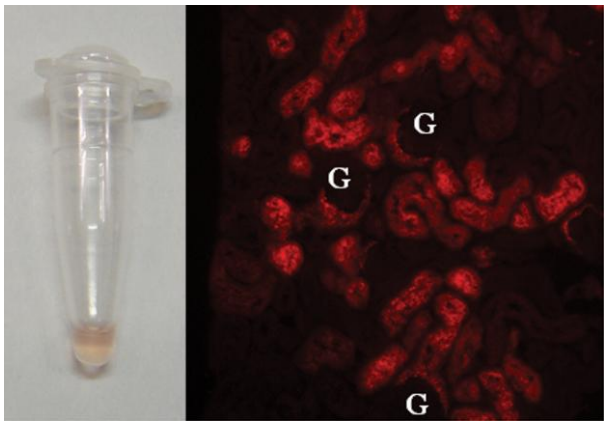

Figure 2. Kidney protection by a red protein.

Ngal protein shows a red color when ligated with iron and siderophore (left). Intravenous injection of A568-labeled Ngal (red fluorescence, right) into normal mice results in rapid glomerular (G) filtration and reabsorption by proximal tubules. Administration of iron-loaded Ngal protects the kidney from renal ischemia-reperfusion injury.

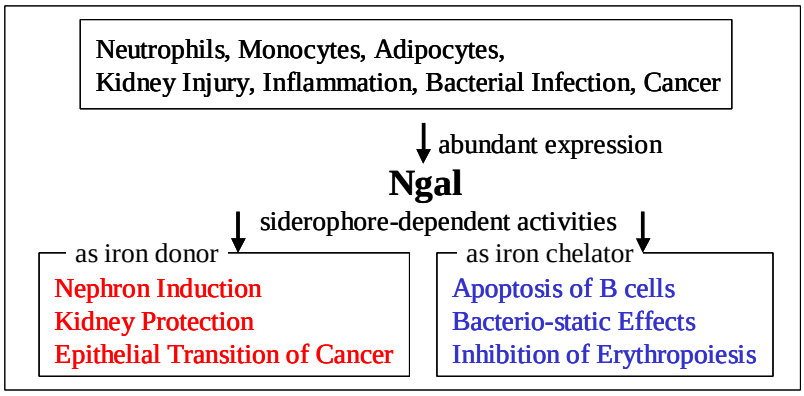

Figure 3. Summary of expressional regulation and biological activities of Ngal.